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Short Communication

Development of novel microsatellite markers for conservation genetic studies of *Vulpes vulpes* (Canidae) by using next-generation sequencing method

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ABSTRACT. The red fox, *Vulpes vulpes* (Canidae), is the most widely distributed terrestrial carnivore worldwide, but this species is classified as endangered in Korea. In this study, we developed 25 polymorphic microsatellite markers that included 3-13 (mean = 6.32) alleles per locus using 22 red fox individuals. The most polymorphic locus was *FR*(*59*)*TG* (13 alleles) and the least polymorphic loci were *FR*(*70*)*TG* and *FR*(*182*)*AG* (3 alleles each). No significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$) was observed for the 25 markers. Observed (H_O) and expected (H_E) heterozygosity varied from 0.182 to 1.000 and from 0.175 to 0.929, respectively. These newly developed microsatellite markers will be useful for investigating the genetic diversity and population genetic structure of *V. vulpes* and will aid in

developing conservation strategies for this species.

Key words: Next-generation sequencing; Endangered species; Microsatellites; Red fox; *Vulpes vulpes*

INTRODUCTION

The red fox (*Vulpes vulpes*, Canidae) is a small carnivorous mammal that is widely distributed in Eurasia, northern Africa, and the Americas, and its status is generally stable across most of its range (IUCN, 2013). In South Korea, however, illegal killing to protect livestock or for fur, combined with habitat loss and fragmentation, have resulted in significant decline in the red fox population (Won and Smith, 1999). In the 1980s, the red fox was thought to be extinct and was listed as an "Endangered Species I" on the Korean Red List (Ministry of the Environment of Korea, 2005). With the exception of a dead red fox discovered in Yanggu, Gangwon Province in 2004, wild *V. vulpes* have not been observed in South Korea since the 1980s. A "Red Fox Restoration Project" was initiated in 2011 by the Ministry of the Environment of Korea. Recently, Yu et al. (2012) suggested that the introduction of individuals from genetically and geographically close northeastern populations, such as from North Korea, China, and Russia, would provide the best alternative for restoring *V. vulpes* in South Korea.

Here, we developed 25 polymorphic microsatellite markers to facilitate conservation and restoration of red fox in South Korea using next-generation sequencing (NGS). Twenty-two individuals of *V. vulpes* from North Korea and northeastern China were used to characterize these microsatellite markers.

MATERIAL AND METHODS

Total genomic DNA was isolated from blood, muscle tissue, or hair of the fox specimens using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer protocol. DNA quality was assessed by electrophoresis on a 1% agarose gel followed by evaluation using a spectrophotometer (Nanodrop, Technologies, Wilmington, DE, USA). To search for microsatellite fragments in *V. vulpes*, we used one DNA sample from Yanggu-gun, Gangwon Province in South Korea. Approximately 5-10 μg genomic DNA was sequenced using one-quarter of a Roche GS-FLX-454 plate (454 Life Sciences, Branford, CT, USA) with a titanium platform, at the National Instrumentation Center for Environmental Management (NICEM, Seoul, South Korea).

The NGS data yielded 206,687 reads in FASTA format and a total of 71,115,299 base pairs (bp) from the genomic DNA of *V. vulpes*. After trimming off low-quality sequences, 174,425 reads remained (65,562,274 bp). From these sequences, we chose 200 with higher copy numbers (c6) of di- and trinucleotide repeats for the amplification test and assessment of polymorphism. A primer set was designed for the flanking sequences of the microsatellite repeat motifs using Primer 3.0 (Rozen and Skaletsky, 2000). The optimal primer size was set to 18-26 bp, and an M13 sequence tag (M13-TGTAAAACGACGGCCAGT) was added to the 5'-end of the forward primer to allow fluorescent labeling during amplification (Schuelke, 2000).

PCR amplifications were performed on all 22 individuals in 20 μL volumes containing 30-50 ng template DNA, 0.5 μL dNTPs (20 mM), 1 μL 10X PCR buffer containing 25 mM $MgCl_2$ (Takara, Japan), 0.25 μL forward and 1 μL reverse primers (8 pM each), and 1 μL fluorescently labeled M13 primer (8 pM; labeled with 6-FAM, VIC, PET, and NED, Ap-

plied Biosystems, Foster City, USA). The amplifications were conducted using the following cycling conditions: 5 min pre-denaturation at 94°C followed by 30 cycles of 30 s at 94°C, 45 s at 56°C, and 45 s at 72°C, followed by 8 cycles of 30 s at 94°C, 45 s at 53°C, and 45 s at 72°C, and then a final 20 min extension step at 72°C.

The fluorescently labeled PCR products were genotyped on an ABI 3710XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) using LIZ-500 as the internal size standard (Applied Biosystems, Foster City, CA, USA), and were visualized using the GeneMarker program (version 2.40, Softgenetics LLC). The data were checked for null alleles and scoring errors using Micro-Checker version 2.2.3 (van Oosterhout et al., 2004). Allele number per locus, observed (H_O) and expected (H_E) heterozygosity, and deviation from Hardy-Weinberg equilibrium (*HWE*) were analyzed using Arlequin version 3.1 (Excoffier et al., 2007).

RESULTS AND DISCUSSION

A total of 200 microsatellite primers were screened in the 22 *V. vulpes* individuals for amplification and polymorphism; 25 microsatellite markers were polymorphic, with the number of alleles per locus ranging from 3 to 13 (mean = 6.32). The H_O and H_E values ranged from 0.182 to 1.000 and 0.175 to 0.929, respectively (Tables 1 and 2). The remaining microsatellite markers were monomorphic or failed to amplify scorable products. No significant ($P < 0.05$) deviations from *HWE* were observed in locus-pair/populations in the 25 newly developed microsatellite markers. In addition, Micro-Checker analysis revealed no evidence for null alleles, scoring error, stuttering, or allelic dropout in any of the loci. Based on these results, we expect that the 25 newly developed microsatellite markers will be useful for estimating genetic diversity and will aid in genetic breeding, monitoring molecular ecology, and conserving genetic resources in *V. vulpes*.

Table 1. Information on the 25 microsatellite loci developed for *Vulpes vulpes*.

Locus	Repeat motif	Fluorescence label	M13 tail +F-primer (5'→3')	R-primer (5'→3')	GenBank accession No.
1 RF(21)TG	$[TG]_n$	6-FAM	TGTAAAACGACGGCCAGTGGAGGCATTTTATTCCATCATA	TCGTGATCTCTCTTAAAGGTGC	KJ588476
2 RF(56)TG	$[TG]_n$	PET	TGTAAAACGACGGCCAGTAATCAGGGGTCTTTTTCTCAAT	ACATCTGAAACCAAATGTTATATGTC	KJ588477
3 RF(59)TG	$[TG]_n$	VIC	TGTAAAACGACGGCCAGTGCTTGTTTTGAAGCTAGGAAGA	TCCCAAAGGAAAAGTAGACTCTC	KJ588478
4 RF(70)TG	$[TG]_n$	NED	TGTAAAACGACGGCCAGTAAAAGAGGGCTACCCTCACTAC	CTGTGTGTCAGCTTATTTGGAA	KJ588479
5 RF(71)TG	$[TG]_n$	VIC	TGTAAAACGACGGCCAGTTTCTAACTCAAAGGTGGCAAAT	TATTACTACCTCTCCCCTCCGT	KJ588480
6 RF(125)CA	$[CA]_n$	6-FAM	TGTAAAACGACGGCCAGTAGGCAAGGCTTTTAACAACATA	ACAAATTAAAACTTCCCCAGGT	KJ588481
7 RF(127)CA	$[CA]_n$	VIC	TGTAAAACGACGGCCAGTACATCAAAGCTACCAACAGAGG	ACAAGAAACAGAATCATGGGAC	KJ588482
8 RF(131)CA	$[CA]_n$	VIC	TGTAAAACGACGGCCAGTCCAGGTATTTACCCAAGTGAAA	GCAGGAATACTCCATTGTTGTT	KJ588483
9RF(136)CA	$[CA]_n$	PET	TGTAAAACGACGGCCAGTTAATTCAGGGCATGGTTTACTC	CCAGGGATACCTGAAGTTGTAA	KJ588484
10 RF(139)CA	$[CA]_n$	VIC	TGTAAAACGACGGCCAGTTAATTATTTGGTAGGCAGGGTG	TGAATTGTGAATGATAGCTTGC	KJ588485
11 RF(143)CA	$[CA]_n$	VIC	TGTAAAACGACGGCCAGTTGGTGTACATCTCTGCTTCACT	CATTTACCAGTCATTGGATGTG	KJ588486
12 RF(147)CA	$[CA]_n$	VIC	TGTAAAACGACGGCCAGTGCAATCATGTGTGTTGAATAGG	TTCCCTACGTACTGCTTTTGTT	KJ588487
13 RF(155)CA	$[CA]_n$	VIC	TGTAAAACGACGGCCAGTATGTTTTTCTTCCAGTGCTTGT	CTTCACTTCCAGAATACCTCCA	KJ588488
14 RF(156)CA	$[CA]_n$	PET	TGTAAAACGACGGCCAGTTTCCCTAGAAGAACGTGACCTA	GATTTCCTCACCTTGTGAACAT	KJ588489
15 RF(157)CA	$[CA]_n$	6-FAM	TGTAAAACGACGGCCAGTCAATTGGCTTATCCTTCAAAAC	CTTCCTTAAGTCTCCCATTCCT	KJ588490
16 RF(162)ATC	$[ATC]_n$	NED	TGTAAAACGACGGCCAGTTACCATTACGTTAGGGATTTGG	CCTCTGTGTTCATTCCAGAAGT	KJ588491
17 RF(163)ATC	$[ATC]_n$	VIC	TGTAAAACGACGGCCAGTCTTCCTTCCCTCATTTCTTTCT	TGTATGCCATATAGTAGGTGCTTT	KJ588492
18 RF(165)TGA	$[TGA]_n$	6-FAM	TGTAAAACGACGGCCAGTGGCTTTGTAAGGAAGAGCATAA	CGCTGCTCTCAGAATAGAGAAC	KJ588493
19 RF(174)GA	$[GA]_n$	NED	TGTAAAACGACGGCCAGTATCAATGAAAGTGAGAGCTGGT	TTTTAGGTTGTTCTATTTCTTGCC	KJ588494
20 RF(176)AG	$[AG]_n$	PET	TGTAAAACGACGGCCAGTTTTGATCTGCACTTTTTCAGTG	TGGCGTCAATAGTTTAAGTGTG	KJ588495
21 RF(182)AG	$[AG]_n$	NED	TGTAAAACGACGGCCAGTTCAAGTTATGGATGGACACAGA	TTAGAATTCAGATAGCCCCTGA	KJ588496
22 RF(195)AAT	$[AAT]_n$	VIC	TGTAAAACGACGGCCAGTACTTGCAGGTGCCCTTATG	TGTTTGTGCTTACTGTCAGAGG	KJ588497
23 RF(198)AAT	$[AAT]_n$	NED	TGTAAAACGACGGCCAGTGCCCACACTCTGATCTGTATCT	AAAGGTTTGAGTTTGAAGGGTT	KJ588498
24 RF(199)AAT	$[AAT]_n$	VIC	TGTAAAACGACGGCCAGTTGCTTGATTTCAACTTAGGTCA	AACATGAGCTTAAACTCCCAAC	KJ588499
25 RF(200)AG	$[AG]_n$	PET	TGTAAAACGACGGCCAGTCTCTCTCAACCTTTCTCCTGAA	TCTGCTTCCACTCAGATCATAA	KJ588500

Table 2. Characterization of 25 microsatellite loci for *Vulpes vulpes*.

Locus	No. of individuals	N_A	Allele size (allelic range)	H_O	H_E	*pHWE*
RF(21)TG	22	6	228-241 (13)	0.818	0.816	0.800
RF(56)TG	22	5	281-289 (8)	0.727	0.709	0.846
RF(59)TG	22	13	257-287 (30)	0.909	0.929	0.779
RF(70)TG	22	3	263-269 (6)	0.409	0.415	0.460
RF(71)TG	22	5	345-325 (10)	0.682	0.708	0.497
RF(125)CA	22	6	151-161 (10)	0.500	0.569	0.435
RF(127)CA	22	7	214-226 (12)	0.864	0.809	0.684
RF(131)CA	22	8	188-203 (15)	0.545	0.555	0.296
RF(136)CA	22	4	238-244 (6)	0.682	0.597	1.000
RF(139)CA	22	4	222-234 (12)	0.318	0.289	1.000
RF(143)CA	22	7	230-242 (12)	0.591	0.651	0.096
RF(147)CA	22	4	285-293 (8)	0.409	0.453	0.470
RF(155)CA	22	11	279-309 (30)	0.864	0.882	0.627
RF(156)CA	22	10	322-342 (20)	0.818	0.871	0.247
RF(157)CA	22	12	280-322 (42)	0.864	0.863	0.974
RF(162)ATC	22	6	217-229 (12)	1.000	0.769	0.215
RF(163)ATC	22	5	260-271 (11)	0.364	0.461	0.061
RF(165)TGA	22	6	278-292 (14)	0.682	0.696	0.592
RF(174)GA	22	5	219-244 (25)	0.182	0.175	1.000
RF(176)AG	22	5	241-249 (8)	0.500	0.564	0.278
RF(182)AG	22	3	231-235 (4)	0.227	0.280	0.431
RF(195)AAT	22	5	253-268 (15)	0.636	0.628	0.815
RF(198)AAT	22	5	254-266 (12)	0.636	0.773	0.487
RF(199)AAT	22	7	272-289 (15)	0.727	0.687	0.353
RF(200)AG	22	6	306-320 (14)	0.727	0.736	0.777

N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; *pHWE* = P value for Hardy-Weinberg equilibrium tests for each marker. The size ranges include 18 base pairs of the M13 tail.

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